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EXHIBIT A

Efficacy of Oncolytic Herpesvirus NV1020 Can Be Enhanced by Combination with Chemotherapeutics in Colon Carcinoma Cells

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ABSTRACT

NV1020, an oncolytic herpes simplex virus type 1, can destroy colon cancer cells by selectively replicating witbin these cells, while sparing normal cells. NV1020 is currently under investigation in a clinical phase I/II trial as an agent for the treatment of colon cancer liver metastases, in combination with conventional chemotherapeutic agents such as 5-fluorouracil (5-FU), SN38 (the active metabolite of irinotecan), and oxaliplatin. To study the synergy of NV1020 and chemotherapy, cytotoxicity and viral replication were evaluated *in vitro* by treating various human and murine colon carcinoma cell lines, using a colorimetric viability assay, a clonogenic assay, and a plaque-forming assay. *In vivo* experiments, using a subcutaneous syngeneic CT-26 tumor model in BALB/c mice, were performed to determine the efficacy of combination therapy. *In vitro* studies showed that the efficacy of NV1020 on human colon carcinoma cell lines HT-29, WiDr, and HCT-116 was additively or synergistically enhanced in combination with 5-FU, SN38, or oxaliplatin. The sequence of application was not important and effects were still apparent after a 21-day incubation period. Three intratumoral treatments with NV1020 (1×10^7 plaque-forming units), followed by three subcutaneous treatments with 5-FU (50 mg/kg), resulted in substantially higher inhibition of tumor growth and prolongation of survival compared with monotherapies (NV1020/5-FU vs. NV1020, $p = 0.027$). On WiDr cells, reduced replication of NV1020, in combination with 5-FU, indicated that additive and synergistic effects of combination therapy must be independent from viral replication. These results suggest that NV1020, in combination with chemotherapy, is a promising therapy for treating patients with metastatic colorectal cancer of the liver. We hypothesize that infection of cells with NV1020 sensitizes the infected cells for the cytotoxic effect of the chemotherapeutics.

OVERVIEW SUMMARY

NV1020, an oncolytic herpes simplex virus type 1, can destroy colon cancer cells by selectively replicating within these cells, while sparing normal cells. Combination therapy with NV1020 and 5-FU, SN38 (the active metabolite of irinotecan), or oxaliplatin showed additively and synergistically enhanced effects in different human colon carcinoma cell lines *in vitro*. The effects were not dependent on the sequence of application and lasted up to 21 days after treatment. In a subcutaneous syngeneic CT-26 tumor model in BALB/c mice, treatment with NV1020 and 5-FU showed substantially higher inhibition of tumor growth and prolongation of survival compared with monotherapies. Further *in vitro* studies showed that viral replication is reduced in the presence of chemotherapeutics and that effects of combination therapy must be independent from viral replication. We hypothesize that NV1020 sensitizes the infected cells for the cytotoxic effect of the chemotherapeutics.

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INTRODUCTION

With an estimated 300,000 new cases per year and about 200,000 deaths, colorectal carcinoma is the third most common malignancy and the second leading cause of cancer deaths in the United States and Europe (Midgley and Kerr, 1999; Viale *et al.*, 2005). About 30% of patients with colorectal cancer will develop metastases. The median survival rate of patients with metastatic colorectal cancer is 15–20.5 months and most patients develop progressive disease and require second-line chemotherapy (Reid *et al.*, 2005).

Metastatic colorectal carcinoma is currently being treated with first-line chemotherapy, including 5-fluorouracil (5-FU) combined with leucovorin (LV), oxaliplatin, and irinotecan. A combination of 5-FU with oxaliplatin, or irinotecan, showed superior efficacy compared with monotherapies (Braun *et al.*, 2004). In 2004, bevacizumab (Avastin), a monoclonal antibody against vascular endothelial growth factor (VEGF) showing increased overall survival in colorectal cancer in combination with 5-FU/leucovorin/irinotecan chemotherapeutic treatment, was approved for the market (Marshall, 2005; Jain *et al.*, 2006). Cetuximab (Erbitux) is a recombinant human/mouse chimeric epidermal growth factor receptor (EGFR) monoclonal antibody. It was approved by the U.S. Food and Drug Administration in February 2004, to be used in combination with irinotecan for the treatment of EGFR-expressing, metastatic colorectal cancer in patients who had failed to improve with irinotecan-based chemotherapy (Wong, 2005). Cetuximab has shown considerable activity, both as a monotherapy and in combination with chemotherapy, in the treatment of metastatic colorectal cancer that is resistant to chemotherapy. At present, the median survival for patients receiving second-line therapy is about 6–8 months and, therefore, further novel therapies, in addition to a combination of chemotherapeutics, or a combination of chemotherapy with antibodies, are needed. All chemotherapeutics used in this study intervene with DNA synthesis: 5-FU is a nucleotide analog that inhibits thymidylate synthase, irinotecan interacts with topoisomerase I, and oxaliplatin is a platinum derivative that intercalates with DNA. In this respect, oncolytic viruses offer a different mode of action for the eradication of cancer cells.

Replication-selective herpesviruses are being developed as anticancer therapies (for review see Varghese and Rabkin, 2002; Ries and Brandts, 2004). NV1020 is a multimutated type 1 herpes simplex virus (HSV-1), with a deletion of *UL24* and the thymidine kinase gene (*tk*), a deletion of one γ34.5, and a 15-kb deletion over the region jointly shared between the unique short (US) and unique long (UL) regions. A 5.2-kb fragment of HSV-2 DNA containing a *tk* gene was reinserted, ensuring susceptibility to antiviral chemotherapy (Meignier *et al.*, 1988). These deletions favor replication of NV1020 in cancer cells, where certain viral genes are not required to counteract cellular antiviral mechanisms. In contrast to tumor cells, a clearly reduced replication of attenuated herpesvirus was shown for G207 in normal human peritoneal mesothelial cells (Coukos *et al.*, 2000) and for rRp450 in normal primary human hepatocytes (Pawlik *et al.*, 2002).

Infection with NV1020 has been shown to cause lysis of a variety of cancer cells, such as those of the pancreas (McAuliffe *et al.*, 2000), prostate (Advani *et al.*, 1999; Cozzi *et al.*, 2002), and head and neck (Wong *et al.*, 2001), as well as gastric cancer cells (Bennett *et al.*, 2002). In pancreatic cancer (McAuliffe *et al.*, 2000), human prostate cancer (Cozzi *et al.*, 2002), hormone-resistant prostate cancer (Advani *et al.*, 1998), chemotherapy/radiotherapy-resistant epidermoid cancer (Advani *et al.*, 1999), and gastric cancer (Bennett *et al.*, 2002) preclinical animal models, NV1020 was shown to be a potential oncolytic agent. It was also demonstrated that NV1020 was efficacious in a murine liver metastasis model of colon cancer (Delman *et al.*, 2000). The study by Advani *et al.* (1999) showed that, in combination with radiation, NV1020 enhanced tumor regression in prostate adenocarcinoma.

A combination of oncolytic virus, such as G207, HSV-1716, or NV1066, with chemotherapeutic agents was shown to be more efficacious than treatment with the virus alone. After G207 was combined with cisplatin, or HSV-1716 with mitomycin C, synergistic effects were observed *in vitro* and *in vivo* (Chahlavi *et al.*, 1999; Toyoizumi *et al.*, 1999; Post *et al.*, 2004; Eisenberg *et al.*, 2005).

MediGene (Martinsried/Planegg, Germany) is currently investigating a combination of NV1020 and standard therapy, including conventional chemotherapeutics, in a phase I/II clinical trial to treat liver metastases of colon cancer. To evaluate the effect of combination therapy *in vitro*, NV1020 was tested on several colon carcinoma cell lines in combination with 5-FU, SN38 (the active metabolite of irinotecan), and oxaliplatin. In addition, a subcutaneous CT-26 tumor model in BALB/c mice was used to evaluate the efficacy of combination therapy. The data presented show that a combination of NV1020 with chemotherapeutics leads to superior treatment effects compared with single therapy *in vitro* and *in vivo*.

MATERIALS AND METHODS

Virus

NV1020 is a derivative of R7020, an attenuated replication-competent virus based on HSV-1 strain F, and is described elsewhere (Meignier *et al.*, 1988, 1990). The virus was propagated on Vero cells and titered by standard plaque assay.

Cell lines

All media and medium supplements were purchased from Invitrogen (Karlsruhe, Germany). Fetal calf serum (FCS) was heat inactivated. CACO-2 cells (German Collection of Microorganisms and Cell Cultures [DSMZ], Braunschweig, Germany) were cultivated in minimal essential medium (MEM) containing 20% FCS, 2 m*M* L-glutamine, and 100 μ*M* nonessential amino acids. HCT-116 (European Collection of Cell Cultures [ECACC], Taufkirchen, Germany) and HT-29 human colon carcinoma cells (DSMZ) were cultured in McCoy's 5A medium with 10% FCS and 2 m*M* L-glutamine. SW480 (DSMZ) and SW620 human colon adenocarcinoma cells (ECACC) were cultured in Leibovitz-15 (L-15) medium containing 10% FCS and 2 m*M* L-glutamine. LoVo human colon adenocarcinoma cells (American Type Culture Collection [ATCC], Manassas, VA) were cultured in Ham's F12K medium with 10% FCS and 2 m*M* L-glutamine. LS174T (ECACC) and WiDr human colon carcinoma cells (ECACC) were cultured in

MEM containing 10% FCS, 2 m*M* L-glutamine, and 1 m*M* sodium pyruvate. Vero African Green Monkey kidney cells (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5 g/liter), 10% FCS, and 2 m*M* L-glutamine. CT-26 murine colon carcinoma cells (Promochem, Wesel, Germany) were cultured in RPMI 1640, 25 m*M* HEPES with 10% FCS, 2 m*M* L-glutamine, and 1 m*M* sodium pyruvate.

Chemotherapeutics

5-Fluorouracil (5-FU) was purchased from Sigma (Taufkirchen, Germany) and dissolved in dimethyl sulfoxide (DMSO, 500 m*M*) for *in vitro* experiments. For *in vivo* experiments, 5-FU (50 mg/ml) was purchased from Medac (Wedel, Germany). SN38, the active metabolite of irinotecan, was a kind gift from Sanofi-Aventis (Paris, France) and was dissolved in DMSO (2.55 m*M*, 1 mg/ml). Oxaliplatin (Alexis Biochemicals, Lausen, Switzerland) was dissolved in water (5 mg/ml, 12.6 m*M*).

Establishment of CT-26 cells resistant to 5-FU: CT-26-5-FU cells

CT-26 cells were first cultivated in medium containing 0.2 μM 5-FU. Selection was then performed by raising the 5-FU concentration in 0.2-μM steps each time cells showed a growth rate comparable to the parental cell line. The selection process was performed for approximately 8 months, until cells showed resistance to 5-FU after the addition of 0.1–0.3 μM 5-FU (see Fig. 2).

Cell viability assay performed on colon carcinoma cell lines

Cells were seeded in a 96-well flat-bottom plate at the following densities: SW480 at 2×10^4 per well, SW620 at 1×10^4 per well, LoVo at 5×10^3 per well, WiDr at 1.5×10^3 per well, LS174T at 2×10^3 per well, CACO-2 at 2×10^3 per well, HT-29 at 2.5×10^3 per well, HCT-116 at 1.25×10^3 per well, and CT-26 at 3.2×10^2 per well. After 22–24 hr of incubation at 37°C in 5% CO_2, cells were infected with NV1020 at concentrations between 10 and 1×10^6 plaque-forming units (PFU)/ml and were further incubated for 2 days. On day 2 after infection, a 1/10 volume of WST-1 agent (Roche Diagnostics, Mannheim, Germany) was added to each well and the cells were incubated at 37°C in 5% CO_2 for up to 4 hr. Absorbance was measured at 450 nm and at a reference wavelength of 655 nm, using an enzyme-linked immunosorbent assay (ELISA) reader. To calculate relative cell growth, untreated cells were defined as 100%.

To determine cell viability, the 5-FU-resistant cell line (CT-26-5-FU) and the parental cell line (CT-26) were seeded at 1.8×10^2 per well (see Fig. 2A and B), or at 1×10^3 per well (see Fig. 2C and D).

Combination of NV1020 and chemotherapeutics

To measure cell viability after treatment with NV1020 and chemotherapeutic agents, cell line HT-29 was seeded in a 96-well flat-bottom plate (1×10^3 cells per well). Cells were preincubated at 37°C in 5% CO_2 for 6 hr. NV1020 or chemotherapeutic agent was then added, resulting in a final concentration as described in Figs. 3 and 4 (see Results). Approximately 24 hr later, supernatant was removed and 100 μl of cell culture medium was added to each well. Furthermore, 100 μl of medium alone, or medium containing NV1020 or chemotherapeutic agent, was added. After 3 days of incubation, a 1/10 volume of WST-1 reagent (Roche Diagnostics) was added to each well. Plates were incubated at 37°C in 5% CO_2 for 4 hr and measured as described above. Thus, a total incubation time of 1 day with virus and 3 days with chemotherapeutic, or vice versa, was determined.

Long-term clonogenic assay

The clonogenic assay is a long-term assay for determining the effect of agents on cells that grow in small colonies. WiDr cells were seeded at a low concentration (80 cells per well in 4 ml of medium) in a 6-well plate. After incubation at 37°C in 5% CO_2 for 5 days, small cell colonies were detectable and cells were treated in 1.5 ml of medium containing NV1020 at a concentration of 1×10^4 PFU/ml, or with 5-FU at a concentration of 100 μM. After incubation overnight, medium was removed and 4 ml of fresh medium was added. Four days after treatment with the virus, cells were treated overnight with the corresponding chemotherapeutic agent, or with NV1020 at the described concentration. Supernatant was removed and fresh medium was added the next day. Medium was changed weekly and colonies were counted 21 days later. Cells were stained and fixed with 1 ml of methylene blue (0.5% methylene blue [w/v] in 70% methanol [v/v]; Merck, Darmstadt, Germany) for 5–10 min at room temperature and washed carefully with deionized water. Thereafter, the colonies were counted. To calculate the additive effect of the treatment, the following calculation steps were performed: (1) the number of untreated colonies was defined as 100% and the percentage of viable cells after virus or 5-FU treatment was calculated for each value; (2) the mean of triplicates and the standard deviation were calculated; and (3) the calculated additive effect (calc add eff) for the combination of NV1020 and 5-FU was determined as calc add eff (%) = (percent viable cells after virus treatment × percent viable cells after 5-FU treatment)/100.

In vitro *assay for determining replication of NV1020*

To determine viral replication in WiDr cells, 3.1×10^4 cells per well were seeded in a 24-well plate. The cells were incubated at 37°C in 5% CO_2 for 6 hr to allow them to adhere. The cells were then infected with either NV1020 at 2×10^4 PFU/ml (virus first and simultaneous treatment), or treated with 10 or 20 μM 5-FU (chemotherapeutic first, simultaneous treatment). The cells were further incubated for 22–24 hr and medium was removed. Next, 10 or 20 μM 5-FU (virus first), virus at 5×10^4 PFU/ml (chemotherapeutic first), or medium (simultaneous treatment) was added. The cells were further incubated for 22–24 hr and medium was changed in all the wells. The cells were incubated for 2 days and the plates were frozen at −80°C. Plates were thawed at 37°C in a water bath and the cell lysate was removed from the wells. Two more freeze–thaw cycles were performed and the lysates were centrifuged and tested in a plaque assay.

Plaque titer assay

Four days before performing the plaque assay, Vero cells were seeded at a density of 5×10^4 cells/cm^2. On the day the plaque assay was to be performed, Vero cells were harvested and seeded, at 7.6×10^5 cells/cm^2 in 1.9 ml of medium per well, in a 6-well plate. Cells were incubated at 37°C in 5% CO_2 for 3–5 hr and then 100 μl of diluted virus was added. Viral samples were diluted in steps of 1:5 or 1:10. A standard virus with a known concentration was diluted to create final concentrations yielding 15–150 plaques per well. Samples were tested in duplicate and incubated for approximately 18 hr. The addition of 100 μl of human gammaglobulin (4% in phosphate-buffered saline [PBS]; Sigma) prevented further spread of the virus and subsequent infection of cells. After a 48- to 56-hr incubation, cells were stained with 1 ml of methylene blue (0.5% methylene blue [w/v] in 70% methanol [v/v]; Merck) for 5–10 min at room temperature and washed with deionized water. Plaques were counted in a range of 15–150 per well and the titer was calculated according to the following formula: titer (PFU/ml) = mean of number of plaques × dilution × 10.

Animals and animal care

Six-week-old, specific pathogen-free, female BALB/c mice (Harlan Winkelmann, Borchen, Germany) were acclimatized for 1 week before being eligible for entry into the study. Mice were housed at five per cage in an individually ventilated cage (IVC) system. Animals were allowed access to sterile pellets (Altromin, Lage, Germany) and tap water *ad libitum*. Animal studies were performed according to federal guidelines for animal care (Tierschutzgesetz BGBI IS 1105) and approved by the Regierung von Oberbayern (Aktenzeichen 209.1-211-2531-92/03).

Syngeneic subcutaneous tumor model

Two independent experiments were performed, comprising a total of 40 animals (10 animals per group) in the first study and a total of 48 animals (12 animals per group) in the second experiment. The second experiment was a repetition of the first with some minor changes made because of time management reasons. For instance, palpation was sometimes performed on different days. The end points of the first and the second studies were day 41 and day 42, respectively. The second animal study was performed to ensure the superiority of the combination therapy, which had already been detected in the first study. BALB/c mice were injected with 1.5×10^5 CT-26 cells in 100 μl of PBS via the right flank. Animals were split into groups of 10 (first experiment) or 12 (second experiment) mice each and treatment was started 8 days later. The first group received three injections per week of PBS–1% glycerin (20 μl), followed by three injections per week of NaCl (0.9%, 100 μl; B. Braun Melsungen, Melsungen, Germany). The second group received three injections per week of NV1020 and three injections per week of NaCl. The third group received three injections per week of PBS–1% glycerin and three injections per week of 5-FU. The fourth group received three injections per week of NV1020 and three injections per week of 5-FU. NV1020 was diluted in PBS–1% glycerin (1×10^7 PFU in 20 μl) and injected into two to four tumor sites on days 0, 3, and 6 after the start of therapy. 5-FU (50 mg/ml in 100 μl) was injected subcutaneously on days 7, 10, and 13, after the start of therapy. The weight of the animals was determined by weighing three randomly chosen animals and averaging the results. The mice were monitored every 2–3 days in order to check their general health and tumor size. Tumor size was determined with a caliper and the calculation ($a \times b \times c/8$) $\times$ π $\times$ (4/3), with a, b, and c being length, width, and depth, respectively. Mice had to be killed when the tumor exceeded 15 mm in diameter, or when tumors ulcerated, because of federal guidelines for animal care.

Analytical and statistical methods

Analysis of combination data by WST-1 assay. To calculate the additive effect of treatment, the mean value of the medium control was subtracted from all other values. Subsequently, the percentage of surviving cells was calculated by reference to untreated cells (defined as 100% viable cells).

For evaluation of the interaction of NV1020 with chemotherapeutic drugs, the software program CombiTool (Fritz Lipman Institute, Jena, Germany; for more detailed information see http://www.imb-jena.de/www_bioc/CombiTool) was used (Dressler *et al.*, 1999). CombiTool is a program designed for the analysis of combination experiments with biologically active agents (Lovat *et al.*, 2000; Vettori *et al.*, 2006). It performs model calculations and an analysis of experimental combination effects for two or three agents, according to both the Loewe additivity (dose additivity) and Bliss independence (independence) criteria. Zero-interaction response surfaces are calculated from single-agent dose–response relations and then compared with experimental data. CombiTool has a built-in graphics facility that allows for direct visualization of response surfaces and contour plots. The contour plots correspond to isobolograms for a number of different effect levels. The CombiTool program offers easy analysis of potential interaction in the complete dose range under study. To transfer experimental data to CombiTool, the experimental growth index was calculated as (1 − percent living cells)/100. The dose–response curves of the single drugs were computed (SigmaPlot 7.0; Systat Software, Point Richmond, CA) according to the following equation:

$$y = A_2 + \frac{(A_1 - A_2)}{1 + \left(\frac{x}{x_0}\right)^p}$$

where $A_1 = 0$ and $A_2 = 1$. This is the four-parameter logistic equation. However, the two parameters A_1 and A_2, which correspond to effects for zero and infinite dose, are assumed to be fixed at 0 and 1. The remaining parameters to be determined from single-agent dose–response relations are x_0, the dose for an effect of 0.5 (50%), and the slope parameter p. The comparison of the response surface to experimental data cannot be evaluated in statistical terms. We consider, therefore, a combination as synergistically interactive if the difference between the predicted dose-additive effect value and the observed combination effect is larger than the 2-fold standard deviation of the experimental combination data. For each of the single drugs, x_0 and p were determined and transferred to CombiTool. In addition, the data representing the experimental effect from the drug combination were transferred to a CombiTool spreadsheet.

The graphs created by CombiTool compare the experimental data with the calculated response surface, which represents the dose-additive effects.

All other statistical analyses were performed with the program SigmaStat 3.1 (Systat Software). The clonogenic assay was analyzed by one-way analysis of variance (ANOVA), using the Tukey test. Survival of animals was compared by log-rank test. In addition, actual changes in tumor volume from baseline (day 8) to end point on day 41/42 were analyzed for the four treatment groups. For the final overall assessment of treatment differences between the treatment groups, all data from the two experiments and from the four subgroups were pooled for one-way ANOVA. To assess superiority between the treatment groups, pairwise comparisons were performed by t tests with and without Dunnett's adjustment. This computation was performed with SAS software, version 8.2 (SAS Institute, Cary, NC).

RESULTS

NV1020 shows a cytotoxic effect on colon carcinoma cell lines

NV1020 was studied in a cell viability assay, testing a panel of eight human colon carcinoma cell lines and one mouse colon carcinoma cell line (CT-26). Exposure to NV1020 resulted in dose-dependent retardation of growth in all cell lines (Fig. 1), indicating that colon carcinoma cells are sensitive to treatment with NV1020.

Colon carcinoma cell lines often become resistant to chemotherapeutics during treatment. Therefore, the cytotoxic effect of NV1020 in a 5-FU-resistant cell line was studied. CT-26, a murine colon carcinoma cell line, was grown in the presence of 5-fluorouracil (5-FU) until reduced sensitivity to the treatment with 5-FU was detected (Fig. 2A). This cell line (CT-26-5-FU) still showed the same sensitivity to treatment with

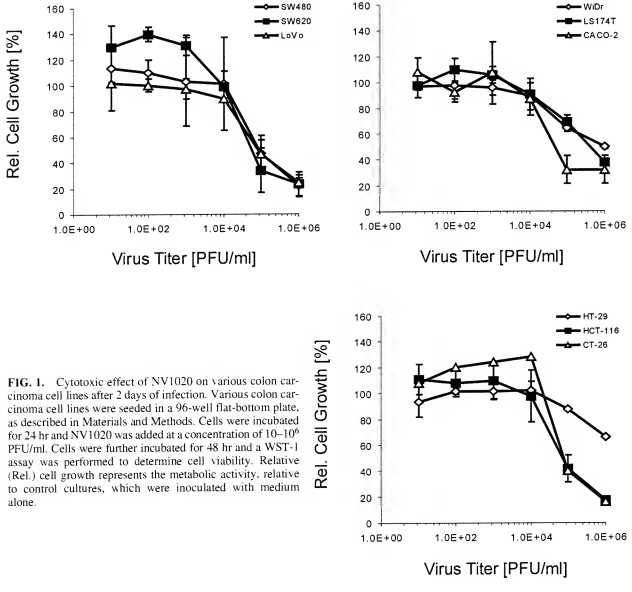


FIG. 1. Cytotoxic effect of NV1020 on various colon carcinoma cell lines after 2 days of infection. Various colon carcinoma cell lines were seeded in a 96-well flat-bottom plate, as described in Materials and Methods. Cells were incubated for 24 hr and NV1020 was added at a concentration of $10–10^6$ PFU/ml. Cells were further incubated for 48 hr and a WST-1 assay was performed to determine cell viability. Relative (Rel.) cell growth represents the metabolic activity, relative to control cultures, which were inoculated with medium alone.

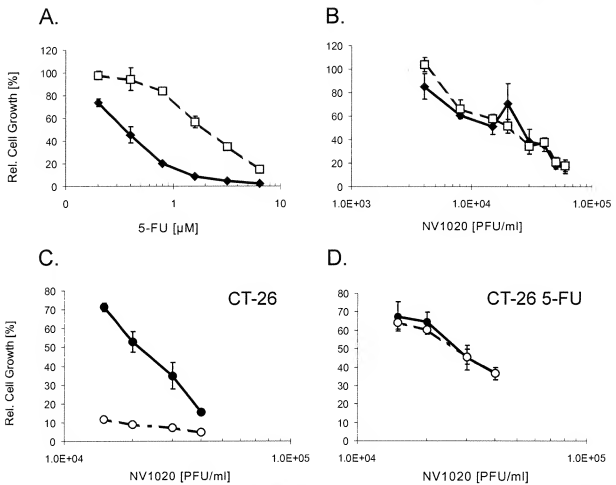
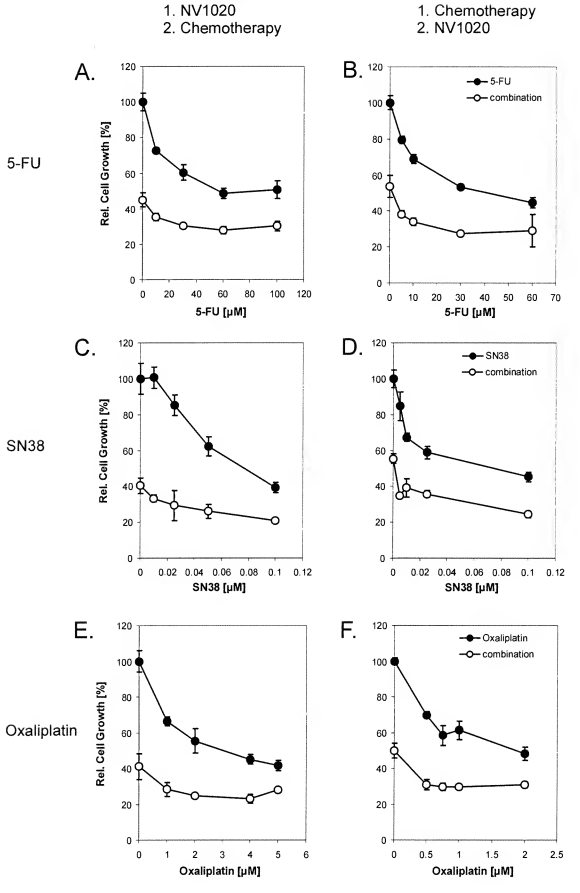


FIG. 2. The 5-FU-resistant cell line CT-26-5-FU is still susceptible to NV1020. (**A** and **B**) Cell lines CT-26 (open squares) and CT-26-5-FU (a 5-FU-resistant derivative of CT-26; solid diamonds) were incubated with 5-FU (**A**) or NV1020 (**B**). 5-FU was added at a concentration of 0.1–6.4 μM (**A**) and NV1020 was added at a concentration of 4×10^3–6×10^4 PFU/ml (**B**). (**C** and **D**) CT-26 cells (**C**) and CT-26-5-FU cells (**D**) were treated with NV1020 alone (solid circles) at a concentration of 1.5×10^4, 2×10^4, 3×10^4, or 4×10^4 PFU/ml. For comparison, cells that were treated with a combination of NV1020 and 0.8 μM 5-FU (open circles) were also tested. For combination treatment, medium containing NV1020 only was changed after 24 hr and then cells were incubated with 5-FU. A WST-1 assay was performed 4 days after addition of the virus. Relative (Rel.) cell growth represents the metabolic activity relative to control cultures, which were inoculated with medium alone.

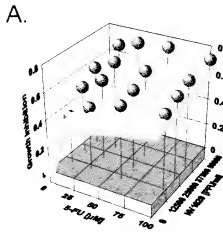
FIG. 3. Superior cytotoxic effect of a combination of NV1020 and chemotherapeutics on HT-29 colon carcinoma cells after 4 days of incubation. HT-29 colon carcinoma cells were seeded in a 96-well flat-bottom plate at 1×10^3 cells per well and incubated for 6 hr. NV1020 (**A**, **C**, and **E**), or chemotherapeutic agent (**B**, **D**, and **F**) was added and cells were incubated for 24 hr. The next day, the medium was completely removed and either the chemotherapeutic agent (**A**, **C**, and **E**), or NV1020 (**B**, **D**, and **F**) was added to the corresponding wells. Cells were incubated for another 3 days and a WST-1 assay was performed to determine cell viability. Depicted are percent viable cells after treatment with a chemotherapeutic agent alone (solid circles) compared with percent viable cells after treatment with a combination of chemotherapeutic agent and NV1020 (open circles). Relative (Rel.) cell growth represents the metabolic activity relative to control cultures, which were inoculated with medium alone. Final concentrations of NV1020 and chemotherapeutic tested were as follows: (**A**) 5×10^4 PFU/ml and 0, 10, 30, 60, and 100 μM 5-FU; (**B**) 1×10^5 PFU/ml and 0, 5, 10, 30, and 60 μM 5-FU; (**C**) 5×10^4 PFU/ml and 0, 0.01, 0.025, 0.05, and 0.1 μM SN38; (**D**) 1×10^5 PFU/ml and 0, 0.005, 0.01, 0.025, and 0.1 μM SN38; (**E**) 5×10^4 PFU/ml and 0, 1, 2, 4, and 5 μM oxaliplatin; (**F**) 1×10^5 PFU/ml and 0, 0.5, 0.75, 1, and 2 μM oxaliplatin.


1. NV1020
2. Chemotherapy
1. Chemotherapy
2. NV1020
A.
B.
C.
D.
E.
F.
5-FU
SN38
Oxaliplatin
Rel. Cell Growth [%]
5-FU [μM]
SN38 [μM]
Oxaliplatin [μM]
combination

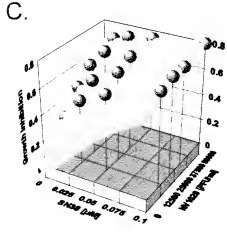
1. NV1020
2. Chemotherapy

1. Chemotherapy
2. NV1020

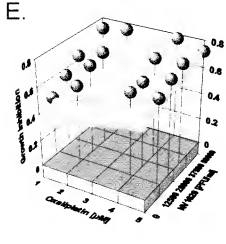
5-FU



SN38



Oxaliplatin



NV1020 (Fig. 2B), indicating that resistance of tumors to chemotherapeutics might not influence the cytotoxic effect of NV1020. CT-26-5-FU cells treated with a combination of NV1020 and 5-FU did not show an enhanced growth-inhibitory effect, compared with treatment with NV1020 alone (Fig. 2D). This can be seen with the original cell line CT-26 (Fig. 2C).

Combination of NV1020 treatment and chemotherapy shows additive to synergistic effects when applied to colon carcinoma cells in vitro

A short-term cell viability assay (WST-1) was performed in order to test the cytotoxic effect of NV1020 in combination with chemotherapeutics. NV1020 was combined with 5-FU, SN38 (active metabolite of irinotecan), or oxaliplatin, and the application started either with NV1020 or with chemotherapeutics. After a 1-day interval, cells were treated with the second agent and incubated until analysis, after a total incubation time of 4 days.

Growth inhibition of HT-29 colon carcinoma cells was clearly stronger at higher concentrations of NV1020 and chemotherapeutic agent (Figs. 3 and 4). Four concentrations of each chemotherapeutic and NV1020 were combined with each other. Combinations of chemotherapeutics with the highest concentration of NV1020 most clearly showed a reduced amount of viable cells compared with treatment only with chemotherapeutic agents (Fig. 3). Because incubation time varied by 1 day, depending on the application schedule, the concentrations of NV1020 or chemotherapeutic agents differed. To show the effects of combination therapy for all combinations tested, growth inhibition was calculated and all values plotted by CombiTool software as a three-dimensional diagram (Fig. 4). This showed additive to synergistic effects on the growth inhibition of HT-29 cells, with no noticeable difference between the different chemotherapeutics being tested. A combination was considered synergistic if the difference between the predicted dose-additive effect value and the observed combination effect was greater than the 2-fold standard deviation of the experimental combination data. This calculation is reflected in the CombiTool diagrams and, in general, a clearer interactive effect could be observed at higher concentrations of both agents (Fig. 4B, D, and F). No difference in growth inhibition was detected if the reversed application scheme was tested (compare left and right panels in Fig. 4). These effects were also observed with colon carcinoma cell lines such as WiDr and HCT-116 (data not shown).

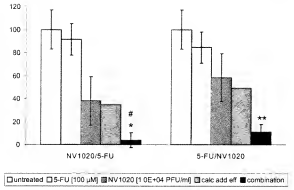


FIG. 5. Synergistic cytotoxic effects of NV1020 and 5-FU on WiDr colon carcinoma cells 3 weeks after incubation. WiDr cells were seeded at a concentration of 80 cells per well in a 6-well plate. After 5 days, cells were treated with either medium, 1×10^4 PFU of NV1020, 100 μM 5-FU, or a combination of both agents. After 3 days, cells were treated with the second agent. Cell colonies were counted after 21 days and the percentage of colonies was compared with untreated cells. The calculated additive effect (calc add eff) was determined by multiplying the percentage of the corresponding monotherapies and dividing by 100. One-way analysis of variance, using the Tukey test, showed the statistical significance of combination therapy: $*p < 0.001$, combination versus 5-FU; $\#p = 0.001$, combination versus NV1020; $**p < 0.001$, combination versus 5-FU and combination versus NV1020.

These data show that the cytotoxic effect of NV1020 and chemotherapeutics on colon carcinoma cells is not affected by the sequence of application. In fact, NV1020 combined with either chemotherapeutic increased growth inhibition additively or synergistically. Likewise, simultaneous addition of chemotherapeutics and NV1020 did not affect the cytotoxicity of NV1020 on colon carcinoma cells and showed additive and synergistic effects (data not shown).

Because the WST-1 assay covers a period of only 4 days, the cytotoxic effect of NV1020 in combination with chemotherapeutics was studied for a longer period of time. The long-term

FIG. 4. Additive and synergistic cytotoxic effects of NV1020 and chemotherapeutics on HT-29 colon carcinoma cells after 4 days of incubation. HT-29 colon carcinoma cells were treated and incubated as described in Fig. 3. The growth index was calculated and results were analyzed with CombiTool software to determine additive, synergistic, or inhibitory effects. Results were plotted in a three-dimensional diagram depicting concentration of NV1020 on the x axis, concentration of chemotherapeutics on the y axis, and growth inhibition on the z axis. To determine the additive effect of single agents, cells were incubated solely with NV1020 or 5-FU. Results were used to calculate values represented by the light gray surface, which represents the theoretical values of an additive effect. The dark gray spheres represent the measured values for combinations tested. Dark spheres above the light gray surface indicate a synergistic effect and dark spheres within the gray surface indicate an additive effect. Spheres below the light gray surface indicate an inhibitory effect of agents on cell viability. Final concentrations of NV1020 and chemotherapeutic tested were as follows: (**A**) 1×10^4, 2×10^4, 3×10^4, and 5×10^4 PFU/ml and 10, 30, 60, and 100 μM 5-FU; (**B**) 1×10^4, 5×10^4, 8×10^4, and 1×10^5 PFU/ml and 5, 10, 30, and 60 μM 5-FU; (**C**) 1×10^4, 2×10^4, 3×10^4, 5×10^4 PFU/ml and 0.01, 0.025, 0.05, 0.1 μM SN38 (**D**) 1×10^4, 5×10^4, 8×10^4, 1×10^5 PFU/ml and 0.005, 0.01, 0.025, and 0.1 μM SN38; (**E**) 1×10^4, 2×10^4, 3×10^4, and 5×10^4 PFU/ml and 1, 2, 4, and 5 μM oxaliplatin; (**F**) 1×10^4, 5×10^4, 8×10^4, and 1×10^5 PFU/ml and 0.5, 0.75, 1, and 2 μM oxaliplatin.

clonogenic assay determines the cytotoxicity of an agent by analyzing the number of colonies after 2–3 weeks, thus showing that the observed effect is not transient. Long-term effects of NV1020 and 5-FU on growth behavior of WiDr cells showed that the cytotoxic effect of combination therapy was equally enhanced if cells were incubated for 21 days after treatment. The percentage of surviving cell colonies after treatment with the combination was lower than the calculated additive effect of single therapies, indicating a synergistic cytotoxic effect (Fig. 5). The sequence of application did not show any different effects, suggesting that 5-FU does not influence the cytotoxic effect of NV1020 and vice versa. A reduction of the number of surviving colonies was also observed with WiDr cells, if NV1020 was combined with SN38 or oxaliplatin (data not shown).

Antitumoral activity of NV1020 in combination with 5-FU in vivo *is superior to single therapy*

Effects of a combination of NV1020 with 5-FU, in comparison with single therapy, were assessed in two independent experiments in a syngeneic subcutaneous mouse model. BALB/c mice harboring a subcutaneous CT-26 tumor were treated with either NV1020 or 5-FU alone, or with NV1020 and 5-FU consecutively. No increase in toxicity was observed after treatment with combination therapy versus buffer or single therapy. For the overall comparison between the treatment groups ANOVA models were used. Initially, three-way and two-way ANOVAs were performed to assess the contribution of treatment, experiment, and sacrifice and their interactions. These analyses showed no statistically significant contribution from the factors experiment and sacrifice as well as the associated interactions. Therefore, both experiments were pooled and survival data and tumor volume were compared. All treatment groups showed superior survival compared with the buffer control group (0 of 22). Treatment with the combination (11 of 22) showed superior survival compared with single therapy (5 of 22 for 5-FU and 6 of 22 for NV1020), which was statistically significant when the combination was compared with NV1020 single therapy ($p = 0.027$; Fig. 6). Statistical significance is based on survival time, because total surviving animals are similar in the 5-FU and NV1020 groups. All treatment groups showed a reduced mean tumor volume compared with the buffer control group. Combination of NV1020 with 5-FU showed a clearly reduced mean tumor volume in comparison with mice treated with a single agent (Table 1). The difference in mean change from baseline tumor volume was compared between control and treatment groups on day 41/42. The largest difference was observed between combination and control groups (Table 2). A significant difference was also found when combination therapy was compared with treatment with 5-FU alone ($p < 0.05$). The number of tumor-free animals was highest after treatment with the combination therapy (7 of 22), compared with treatment with NV1020 (4 of 22) or 5-FU (4 of 22) alone. If animals were tumor free, this was due either to regression, or to prevention of tumor growth at an early stage. Regression of tumors was observed in 2 of 22 animals for NV1020-treated animals, in 1 of 22 animals in the 5-FU group, and in 3 of 22 in the combination group. In two of three animals in the combination group, tumors were only temporarily reduced in size. Prevention of tumor growth was observed in 2 of 22 animals after NV1020 treatment, in 3 of 22 animals after 5-FU treatment, and in 6 of 22 animals after treatment with a combination of both agents. These data confirm the observation, from *in vitro* experiments, that combination of 5-FU with NV1020 does not inhibit the effect of NV1020, but rather enhances the cytotoxic effect.

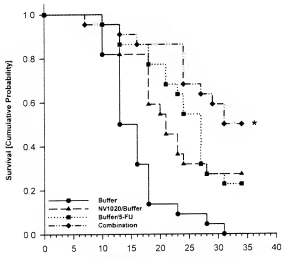


FIG. 6. Survival of mice in a syngeneic subcutaneous tumor model after treatment with NV1020 and 5-FU. BALB/c mice were injected with 1.5×10^5 CT-26 cells via the flank and treatment was started 8 days later, when the tumor was palpable. NV1020 (solid triangles, 1.0×10^7 PFU) was injected at two to four sites into the tumor on day 0, 3, and 6, after the start of therapy. 5-FU (solid squares, 50 mg/kg) was injected subcutaneously on day 7, 10, and 13, after the start of therapy. Treatment of the combination (solid diamonds) and buffer (solid circles) group was likewise applied. Mice were killed when the tumor size exceeded 15 mm in diameter, tumors became ulcerated, or ascites developed. Results were plotted in a Kaplan–Meier plot and analyzed by applying a log-rank test. Statistical significance in survival time was observed between groups treated with combination versus NV1020. *$p = 0.027$, combination versus NV1020.

TABLE 1. MEAN TUMOR VOLUMES ON DAY 41/42 AFTER TUMOR CELL INOCULATION IN SYNGENEIC SUBCUTANEOUS MOUSE MODEL[a]

Group	*Mean tumor volume (mm^3)*	*95% confidence limits (mm^3)*
Control	524.74	444.64, 604.84
5-FU	339.02	243.93, 434.11
NV1020	267.43	179.95, 354.90
NV1020 + 5-FU	153.53	58.35, 248.71

[a]Mean tumor volumes were assessed on day 41/42 after tumor inoculation. Start of therapy was on day 8 after tumor inoculation. If animals had to be killed earlier because of excessive tumor size, tumor volume on the day of sacrifice was taken into account.

TABLE 2. GROUP COMPARISON OF CHANGES FROM BASELINE IN TUMOR VOLUMES ON DAY 41/42 AFTER TUMOR CELL INOCULATION IN SYNGENEIC SUBCUTANEOUS MOUSE MODEL[a]

Group comparison	*Differences between means (mm^3)*	*95% confidence limits (mm^3)*
Control vs. 5-FU[b]	185.72	64.45, 307.00
Control vs. NV1020[b]	257.31	136.04, 378.59
Control vs. NV1020 + 5-FU[b]	371.21	249.93, 492.48
5-FU vs. NV1020 + 5-FU[b]	185.49	64.21, 306.76
5-FU vs. NV1020	71.59	−49.68, 192.87
NV1020 vs. NV1020 + 5-FU	113.89	−7.38, 235.17

[a]Mean tumor volumes were assessed as changes in tumor volume from the start of therapy (day 8) until day 41/42 and compared between groups. If animals had to be killed earlier because of excessive tumor size, tumor volume on the day of sacrifice was taken into account.

[b]Comparisons significant at the 0.05 level.

Combination of 5-FU with NV1020 reduces viral replication in colon carcinoma cells

A possible explanation for the observed synergistic effect is the enhancement of viral replication by chemotherapeutics. To determine viral replication in the presence of chemotherapeutics, viral titer was determined after incubation of WiDr colon carcinoma cells with NV1020 and 5-FU. Sequential application, or simultaneous application of both agents, did not show different results. In all cases, a reduction in viral titer was observed (Fig. 7). Because no effect of 5-FU on cell viability was observed 1 day after incubation, the reduced viral titer is supposedly not caused by a reduction in cell number or cell viability at the time of infection with NV1020. These data were confirmed in other cell lines such as HCT-116, CT-26, and HT-29 (data not shown) and indicate that viral replication is not the cause of the synergistic effect observed after treatment with combination therapy.

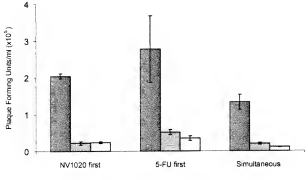


FIG. 7. Viral replication in WiDr cells was reduced if NV1020 was combined with 5-FU. WiDr cells were either first treated with NV1020, or with 5-FU, or with both agents simultaneously. Cells were incubated with the virus alone (dark gray columns), or with NV1020 in combination with 10 μM 5-FU (light gray columns), or with 20 μM 5-FU (open columns). NV1020 at 2×10^8 PFU/ml (NV1020 first, simultaneous treatment) and NV1020 at 5×10^8 PFU/ml (5-FU first) was applied. After 4 days of incubation, cells were harvested and tested for plaque-forming units on Vero cells, using a plaque assay.

DISCUSSION

Oncolytic herpesviruses are under investigation for the treatment of several kinds of diseases, among which is hepatic metastatic colorectal cancer (Varghese and Rabkin, 2002; Delman *et al.*, 2004). The combination of oncolytic virus therapy with chemotherapeutics was previously shown to be advantageous, compared with therapy with either single agent (Chahlavi *et al.*, 1999; Toyoizumi *et al.*, 1999; You *et al.*, 2000). In this paper, we show that NV1020 has a cytotoxic effect on a panel of nine colon carcinoma cell lines tested, among which was a murine cell line (CT-26). A 5-FU-resistant cell line (CT-26-5-FU) was shown to be susceptible to NV1020, indicating that the mode of action of the cytotoxic effect on colon carcinoma cells involves different pathways for 5-FU and NV1020. This hypothesis is further supported by the observation that the treatment of CT-26-5-FU cells with a combination of NV1020 and 5-FU did not show any synergistic effects.

In cell lines that are not resistant to chemotherapeutics such as 5-FU, irinotecan, and oxaliplatin, synergistic effects after treatment with NV1020 and one chemotherapeutic could be observed. These were not only transient, but proved to be effective up to 3 weeks after treatment of the cells. No antagonism was observed. The synergistic effects were not dependent on the cell line, or on the kind of chemotherapeutic being used. Also, the sequence of application did not show any clear preferences. These results are in concordance with studies of human non-small cell lung cancer cells that were treated with mitomycin C (Toyoizumi *et al.*, 1999). The *in vitro* results were further supported by *in vivo* data, using a syngeneic subcutaneous tumor model in BALB/c mice. Prolonged survival after treatment with NV1020 and 5-FU was observed, as well as more tumor-free animals and regression of tumor in three cases.

Enhancement of viral replication by chemotherapeutics leading to increased cell death could be a possible explanation for the detected synergistic effects. This was shown to be the case in several studies (Petrowsky *et al.*, 2001; Eisenberg *et al.*, 2005; Adusumilli *et al.*, 2006). However, in this study, repli-

cation of NV1020 in combination with 5-FU, SN38 (irinotecan), or oxaliplatin was clearly reduced if chemotherapeutics were added before, simultaneously, or after virus treatment. Therefore, enhancement of viral replication cannot account for the synergistic effect observed after combination therapy. Other studies using oncolytic viruses in combination with chemotherapy showed either no effect of chemotherapeutics on viral replication (Heise *et al.*, 2000; Pawlik *et al.*, 2002; Tyminski *et al.*, 2005), or reduced replication of HSV-1726 in combination with higher doses of mitomycin C (Toyoizumi *et al.*, 1999). A study using adenovirus in combination with gemcitabine showed a reduction in the initial rate of viral replication, but the total amount of virus was not affected by the combination (Raki *et al.*, 2005).

We hypothesize that infection of cells with NV1020 sensitizes the infected cells for the cytotoxic effect of the chemotherapeutics—independent of the sequence of application. Because fluorodeoxyuridine (FUdR, a conversion product of 5-FU) was shown to induce cell cycle arrest in the S phase in colorectal cell lines (Petrowsky *et al.*, 2001), one explanation might be that cells are more susceptible to the DNA-damaging effects of 5-FU while arrested in the S phase (due to viral infection).

Another explanation could be that HSV infection can induce the production of molecules such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or interferon-γ (IFN-γ). It was described that infection of immature dendritic cells (DCs) with herpes simplex virus induced expression of TRAIL (Muller *et al.*, 2004). It has been shown that colon carcinoma cells can also produce TRAIL (Strater *et al.*, 2002; Wang *et al.*, 2002). This indicates that in principle HSV may also induce TRAIL expression on colon carcinoma cells. For the replication-restricted herpesvirus mutant HSV-1716, it was described that intratumoral administration of the virus induced expression of IFN-γ in monocytes and DCs (Benencia *et al.*, 2005).

The addition of TRAIL to 5-FU enhanced its cytotoxic effect after treatment of gastric and colon cancer cells, as well as renal cell carcinoma cells (Mizutani *et al.*, 2002; Shimoyama *et al.*, 2002). In renal cell carcinoma cells, TRAIL was shown to enhance the intracellular accumulation of 5-FU and 5-fluorodeoxyuridine monophosphate (FdUMP, a metabolite of 5-FU), both interfering with DNA synthesis. Thus, induction of TRAIL by NV1020 could be an explanation for the enhanced cytotoxicity seen in colon carcinoma cells treated with NV1020 and 5-FU.

IFN-γ might play a role in the so-called "thymineless cell death" that is induced in HT-29 and other colon carcinoma cells after addition of 5-FU. Addition of IFN-γ was shown to enhance cell death by 5-FU, while Fas and Fas ligand played a role in this enhancement (Houghton *et al.*, 1997; Tillman *et al.*, 1999). Thus, NV1020 could induce production of IFN-γ, which in turn induces expression of Fas ligand and Fas, leading to enhanced cell death of 5-FU-treated cells. Because the major source of IFN-γ production is immune cells, this mechanism will probably also be important *in vivo*, where immune cells are present.

In summary, we showed the efficacy of NV1020 in a subcutaneous tumor model using colon carcinoma cells. The oncolytic virus has great potential as an agent for the treatment of colon carcinoma liver metastases. NV1020 did not seem to be influenced by the often occurring resistance to chemotherapeutics, such as 5-FU, and showed additive to synergistic effects in combination with current standard therapy chemotherapeutics.

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